LUPINUS MARIAE-JOSEPHI, A NEW LUPIN ENDEMIC OF SOILS WITH ACTIVE LIME AND HIGH pH IN EASTERN SPAIN, IS NODULATED BY A NEW BACTERIAL LINEAGE WITHIN *BRADYRHIZOBIUM* GENUS*

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ABSTRACT

***Lupinus mariae-josephi* is a recently described species (Pascual, 2004) able to grow in soils with high pH and active lime content in the Valencia province (Spain). *L. mariae-josephi* endosymbionts are extremely slow-growing bacteria with genetic and symbiotic characteristics that differentiate them from *Bradyrhizobium* strains nodulating *Lupinus* spp. native of the Iberian Peninsula and adapted to grow in acid soils. Cross-inoculation experiments revealed that all the endosymbiotic isolates from *L. mariae-josephi* tested are legume-host selective and are unable to nodulate species such as *L. angustifolius*, and *L. luteus*. In contrast, *Bradyrhizobium* strains from *Lupinus* spp. tested were able to nodulate *L. mariae-josephi*, although the nodules fixed nitrogen inefficiently. Phylogenetic analysis was performed with housekeeping genes (*rrn*, *glnII*, *recA*, *atpD*) and nodulation gene *nodC*. Housekeeping gene phylogeny revealed that *L. mariae-josephi* rhizobia form a strongly supported monophyletic group within *Bradyrhizobium* genus. This cluster also includes *B. jicamae* and certain strains of *B. elkanii*. Contrarily, isolates from other *Lupinus* spp. native of the Iberian Peninsula were grouped mainly within *B. canariense* and two *B. japonicum* lineages. Phylogenetic analysis of *L. mariae-josephi* isolates based on the *nodC* symbiotic gene defined a solid clade close to isolates from Algerian *Retama* spp. and to fast-growing rhizobia.**

KEY WORDS

Lupinus mariae-josephi, endosymbiotic bacteria, *Bradyrhizobium,* taxonomic diversity, alkaline soils, lupin endemism

INTRODUCTION

Lupinus is a complex genus comprising *ca.* 275 species. The majority of species are distributed in the New World and only 13 are found in the Old World, of which six are native of the Iberian Peninsula: the yellow lupin *L. luteus*, the narrow-leaf lupin *L. angustifolius*, the blue lupin *L. cosentinii*, *L. hispanicus*, *L. gredensis* (*L. hispanicus* subsp. *bicolor*) and *L. micranthus*, all thriving mainly in acid or neutral soils (pH 5.0–7.0) (Castroviejo and Pascual, 1999). Recently a new endemic species of *Lupinus*, described as *L. mariae-josephi* (Pascual, 2004), has been found in the Eastern Iberian Peninsula. *L. mariae-josephi* is in danger of extinction due to its reduced habitat, now restricted to four geographical locations in the Valencia province: Montserrat, Llombai, Xàtiva and Gandía. The habitat of *L. mariae-josephi* is a singular chromic Luvisol with active lime and high pH (≈ 8.0), also known as "terra rossa". In this regard, *L. mariae-josephi* contrasts with other lupins from the Iberian Peninsula that are adapted to acid or neutral soils. A recent taxonomic study placed *L. mariae-josephi* in a unique phylogenetic position within the *Lupinus* genus (Mahé et al., 2011).

The lupins, like most legumes, are able to establish a nitrogen fixing symbiosis with bacteria collectively

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known as rhizobia. Among rhizobia isolated from lupin nodules, fast-growing strains (poorly characterized) and mainly members of *Bradyrhizobium* genus have been found (Barrera et al., 2003). Lupin bradyrhizobia are a heterogeneous group of rhizobia where most of the isolates from *Lupinus* in Europe, Australia and Africa belong to the *B. japonicum* or the *B. canariense* species and are intermingled with isolates from other *Genisteae* legumes and from *Ornithopus* spp. In contrast, *Lupinus* spp. from the New World are mostly nodulated by strains of *B. japonicum* (Jarabo-Lorenzo et al., 2003, Stepkowski et al., 2007). An initial characterization of *L. mariae-josephi* endosymbionts has been carried out by our group (Sánchez-Cañizares et al., 2011).

Efficient rhizobial strains were isolated using trap-plants from soils of five different sites in the Llombai area (Valencia) and only 1 to 10 nodules were observed per plant, which suggests a low concentration of specific rhizobia in these soils. Thirteen isolates were selected for their efficient nodulation and nitrogen fixation capacity, and were designated as Lmj strains. Unlike other bradyrhizobia isolated from lupins growing in acid soils, all the Lmj strains are extra-slow growing bacteria with a mean generation time greater than 20 h in YMB or ~15 h in AGB (Arabinose Gluconate Broth), although differences were observed among them. Lmj colonies were not mucoid and had a size of <2 mm diameter after 6 days on YMA plates. This extremely slow growth rate complicated phenotypic characterization. Growth experiments at different pH showed that *L. mariae- josephi* isolates are unable to grow at pH 5.0, in contrast to the *B. japonicum* USDA110 strain and isolates from *L. angustifolius*.

SYMBIOTIC PHENOTYPES OF *L. MARIAE-JOSEPHI* STRAINS

Symbiotic properties of the new Lmj strains were investigated by two types of legume cross-inoculation tests. In a first assay, five Lmj isolates were used to inoculate seven *Lupinus* spp. growing in acid soils in the Iberian Peninsula and six other legume species, most of them previously reported to be nodulated by lupin rhizobia (Table 1). In a second assay, twelve strains isolated from the aforementioned *Lupinus* spp. and strains from other legumes were tested for nodulation with *L. mariae-josephi* plants (Table 2).

Regarding the first assay, the five Lmj strains exhibited similar behavior with most of the legumes tested. Efficient red nodules were observed with *L. mariae-josephi* host controls. *L. albus* showed abundant nodules, consistent with the promiscuous nodulation character of this *Lupinus* species. *L. cosentinii* and *L. micranthus* were nodulated only by some of Lmj strains tested, and nodules showed relatively low nitrogenase activity (assayed by acetylene reduction). Absence of nodulation was observed with *L. luteus*, *L. angustifolius*, *L. hispanicus* and *L. gredensis*. Only one Lmj strain was able to produce efficient nodules with *M. atropurpureum* while the other strains were either non-nodulating or produced white tumor-like

Table 1. Legume host range of representative *L. mariae-josephi* isolates (Lmj).

Legume hosts \ Isolates	Lmj A2	Lmj B2b	Lmj C	Lmj Db2	Lmj H2p
L. mariae-joseph	□	□	□	□	□
L. albus	⊙	⊙	⊙	⊙	⊙
L. cosentinii	⊙	⊙	⊙	☒	⊙
L. micranthus	⊙	☒	⊙	☒	⊙
L. angustifolius	☒	☒	☒	☒	☒
L. luteus	☒	☒	☒	☒	☒
L. hispanicus	☒	☒	☒	☒	☒
L. gredensis	☒	☒	☒	☒	☒
Macroptilium atropurpureum	○	○	⊙	ND	○
Vigna sinensis	○	○	○	○	○
Glycine max	☒	☒	☒	☒	☒
Lotus corniculatus	☒	☒	☒	ND	ND
Ornithopus sp.	☒	☒	☒	ND	☒
Cicer arietinum	☒	☒	☒	☒	☒

Nodulation was evaluated by number and colour of nodules: red (□), reddish (⊙), white (○), no nodules (☒). ND not determined.

Table 2. Nodulation of *L. mariae-josephi* inoculated with strains from different hosts.

Strain	Original host	Nodulation of *L. mariae-josephi*
LmjH2p	*L. mariae-josephi*	□
ISLU27	*L. albus*	⊙
ISLU203	*L. albus*	⊙
ISLU12	*L. cosentinii*	○
ISLU9	*L. cosentinii*	⊙
ISLU13	*L. micranthus*	⊙
ISLU122	*L. micranthus*	⊙
ISLU101	*L. angustifolius*	○
ISLU22	*L. angustifolius*	⊙
ISLU8	*L. luteus*	○
ISLU78	*L. luteus*	⊙
ISLU21	*L. hispanicus*	⊙
ISLU40	*L. hispanicus*	○
ISLU16	*Ornithopus compressus*	⊙
UPM861	*Ornithopus compressus*	⊙
USDA8	*Glycine max*	☒
USDA275	*Glycine max*	☒
USDA110	*Glycine max*	☒
NGR234	*Macroptilium atropurpureum*	☒
UPM924	*Vigna sinensis*	○
TONO	*Lotus corniculatus*	☒
UPM835	*Cicer arietinum*	☒

Nodulation was evaluated by number and colour of nodules: red (□), reddish (⊙), white (○), no nodules (☒).

nodules with no nitrogenase activity, as it was also the case with cowpea (*Vigna unguiculata*). Soybean (*Glycine max*) chickpea (*Cicer arietinum*), *Lotus corniculatus,* and serradella (*Ornithopus* spp.) were not nodulated by Lmj strains.

The results obtained using *L. mariae josephi* plants as host showed the induction of inefficient nodules by isolates from *L. albus, L. cosentinii, L. micranthus, L. angustifolius, L. luteus* and *L. hispanicus*, although differences were observed among isolates from the same lupin. On the other hand, the strains isolated from soybean (either *B. japonicum* or *B. elkanii*), cowpea or chickpeas were unable to nodulate *L. mariae-josephi* while serradella strains produced poorly effective nodules (Table 2).

PHYLOGENY OF *L. MARIAE-JOSEPHI* STRAINS

Phylogenetic analyses from partial DNA sequences of four housekeeping genes (16S rRNA, *glnII, recA,* and *atpD*) and nodulation gene *nodC* were carried out. The analyses also included representative isolates of *Lupinus* spp. adapted to growth in acid soils from the Iberian Peninsula, one isolate from serradella and two *B. elkanii* strains, as well as available sequences in the GenBank/EMBL databases from fast-growing strains of rhizobia, and reference or recently isolated strains of *Bradyrhizobium*. Phylogenetic trees were built using both the ML and NJ methods. Consistent results were obtained by both sequence analysis methods and only ML trees are presented in Figures 1, 2 and 3.

Phylogeny based on 16S rRNA gene

The phylogenetic tree based on 16S rRNA gene sequence indicated that all *L. mariae-josephi* strains form a strongly supported (> 90% ML bootstrap) monophyletic Clade (Clade I) within the *Bradyrhizobium* genus (Fig. 1). The group also included *B. jicamae*, some strains of *B. elkanii* (Ramirez-Bahena, 2009), and isolates from Argelian *Retama* spp. (Boulila et al., 2009) and from Peruvian *Phaseolus lunatus* (Ormeño-Orrillo et al., 2006). In contrast, isolates from *Lupinus* spp. such as *L. luteus, L. angustifolius, L. cosentinii, L. hispanicus, L. gredensis* and *L. micranthus* growing in acid soils in different geographical locations of the Iberian Peninsula (ISLU isolates), as well as isolates from *Ornithopus* spp., were grouped within *B. japonicum*-like lineages in Clade II.

Phylogenies based on housekeeping genes glnII, recA, and atpD

Analyses based on concatenated sequences of genes *glnII, recA,* and *atpD* were consistent with the 16S rRNA gene phylogenetic tree, since both divergent clades were again observed (Fig. 2). Clade I included all Lmj isolates and *B. jicamae* PAC68, and was separated from Clade II, that contained the isolates from all the other *Lupinus* spp. from the Iberian Peninsula (ISLU strains). Most of the ISLU isolates grouped with the *B. canariense* reference strain BTA-1, and some with the *B. japonicum* bv *genistearum* lineage. Based on the concatenated (*glnII+recA+atpD*) tree, Lmj isolates form a single monophyletic cluster that includes three sub-groups. These sub-groups are also differentiated by symbiotic properties and could correspond to different genospecies. The individual analyses with *glnII, recA,* and *atpD* produced essentially the same phylogenetic trees with minor differences.

Phylogeny based on symbiotic gene nodC

The signal molecules that trigger nodule formation in legume host plants are called Nod Factors. NodC is an essential protein required for Nod factor production. The tree based on *nodC* gene gathered Lmj strains in a uniform group (Fig. 3) unrelated to *nodC* sequences from other *Lupinus* spp. isolates. The Lmj *nodC* group was

close to *nodC* sequences present in strains from *Retama* spp. from Algeria and in fast growing rhizobia such as *Mesorhizobium mediterraneum*, suggesting that symbiotic genes from Lmj strains and from strains isolated from other *Lupinus* spp. derive from different ancestors.

CONCLUSIONS

The novel *Lupinus mariae-josephi* species thrives in soils with active lime and high pH, in contrast with other *Lupinus* spp. adapted to grow in acid soils. *L. mariae-josephi* nodule isolates are extra-slow growing bacteria sharing this characteristic with isolates from Lima beans (*Phaseolus lunatus*) from Peru and retama (*Retama raetam* and *R. sphaerocarpa*) from Northern Algeria.

The basic nature of the soils where *L. mariae-josephi* thrives could be related to the inability of their endosymbiotic bacteria to grow at pH 5. In this regard, it is significant that *L. mariae-josephi* endosymbiotic bacteria are unable to nodulate *Lupinus* spp. that require acid soils, such as *L. angustifolius*, *L. luteus*, *L. hispanicus* and *L. gredensis*, in contrast with their capacity to nodulate

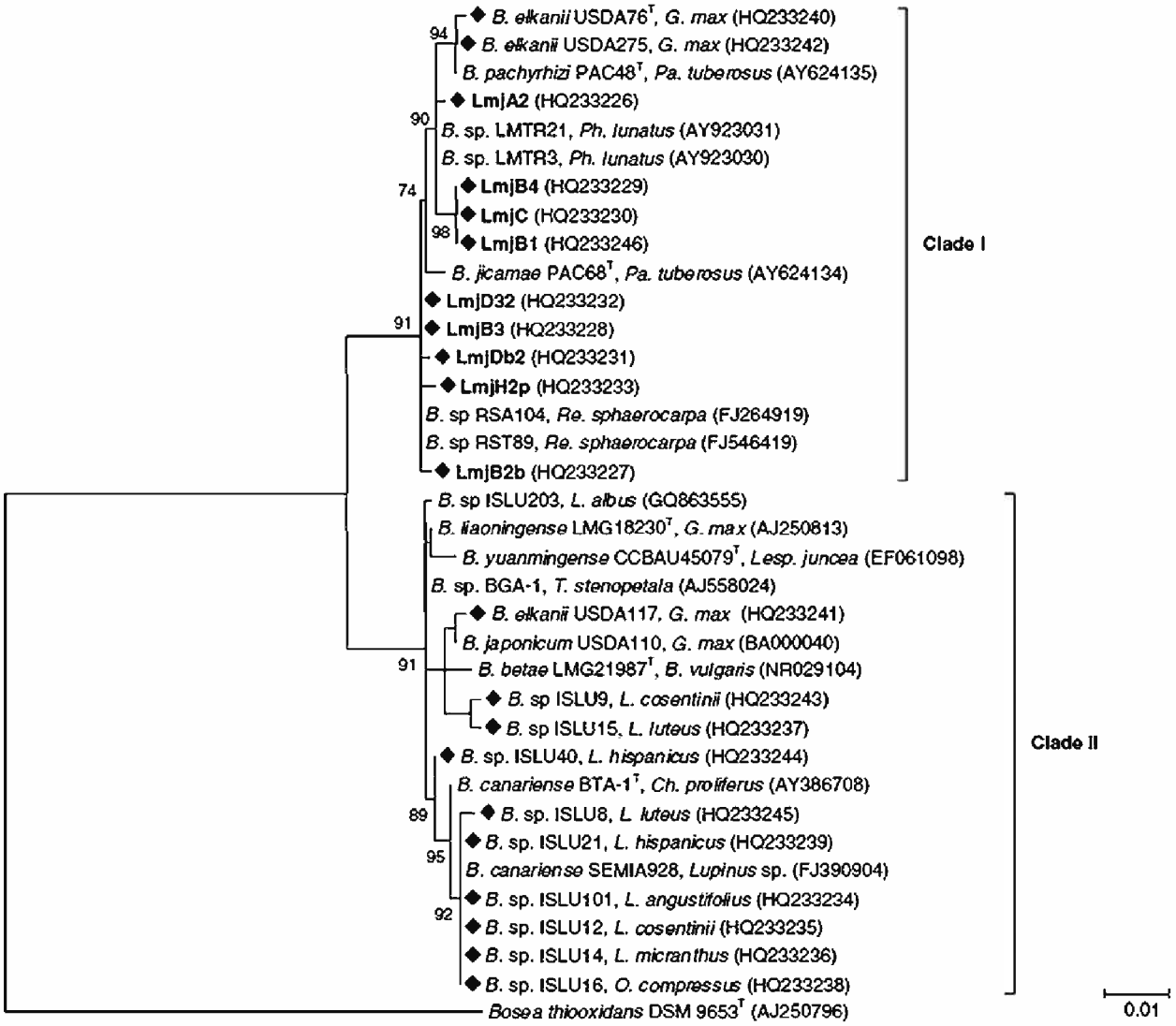

Figure 1. Maximum likelihood phylogenetic tree showing relationships of nodule isolates from *L. mariae-josephi* and other symbiotic bacterial strains based 16S rRNA gene sequences (1400 bp). Strains designated Lmj have been isolated from *L. mariae-josephi* nodules in this study, and are shown in boldface type. Bootstrap values (greater than 70%) were calculated for 1000 subsets and are indicated at the relevant nodes. Isolates of *L. mariae-josephi* and strains indicated with diamonds were sequenced in this study. Legume hosts follow strain names. Accession numbers from GenBank are shown in brackets. The scale bar shows the number of substitutions per site. Abbreviations: A, *Azorhizobium*; B, *Bradyrhizobium*; R, *Rhizobium*; S, *Sinorhizobium*; L, *Lupinus*; O, *Ornithopus*; Re, *Retama*; Lesp, *Lespedeza*; G, *Glycine*; M, *Medicago*; P, *Pisum*; D, *Desmodium*; Ch, *Chamaecytisus*; Se, *Sesbania*; T, *Teline*; Ph, *Phaseolus*; Pa, *Pachyrhizus*.

species that tolerate basic soils such as *L. cosentinii* and *L. albus* This behavior may suggest an effect of soil on the nodulation specificity of *L. mariae-josephi* bacteria, which is relevant, since most, if not all, the *Lupinus* spp. from the Old World also thrive in acid soils. However, it is more likely that the *Lupinus* spp. host is the determinant factor for specificity, and that the capacity of *L. mariae-josephi* bacteria to nodulate *L. albus* and *L. cosentinii* is more consistent with the previously reported promiscuity of these species towards diverse lupin endosymbiotic bacteria.

Phylogenetic analyses of Lmj nodule isolates based on comparison of sequences from "housekeeping genes" (16S rRNA, *glnII, recA, atpD*) yielded convergent results showing that the Lmj nodule isolates group in a new clade within the *Bradyrhizobium* genus, which was well differentiated from the currently named species of *Bradyrhizobium* and, singularly, from the nodule isolates from other *Lupinus* spp. native of the Iberian Peninsula and of other places around the world, all of which are mainly related to *B. japonicum* and *B. canariense* species (Stepkowski et al. 2007). Surprisingly, nodule symbionts of *Retama sphaerocarpa* from Northern Algeria were phylogenetically close to *L. mariae-josephi* isolates, as supported by the phylogenetic analysis of *recA* and *glnII* gene sequences (Sanchez-Cañizares et al., 2011). However, additional data are needed to understand the meaning of this phylogenetic proximity.

According to the phylogenetic *nodC* tree, Lmj isolates belong to a single evolutionary lineage distant from the bradyrhizobia nodulating other *Lupinus* spp. studied so far, which are included in the *B. canariense* or *B. japonicum* lineages. Although analysis of more symbiotic genes is needed, the high degree of similarity among

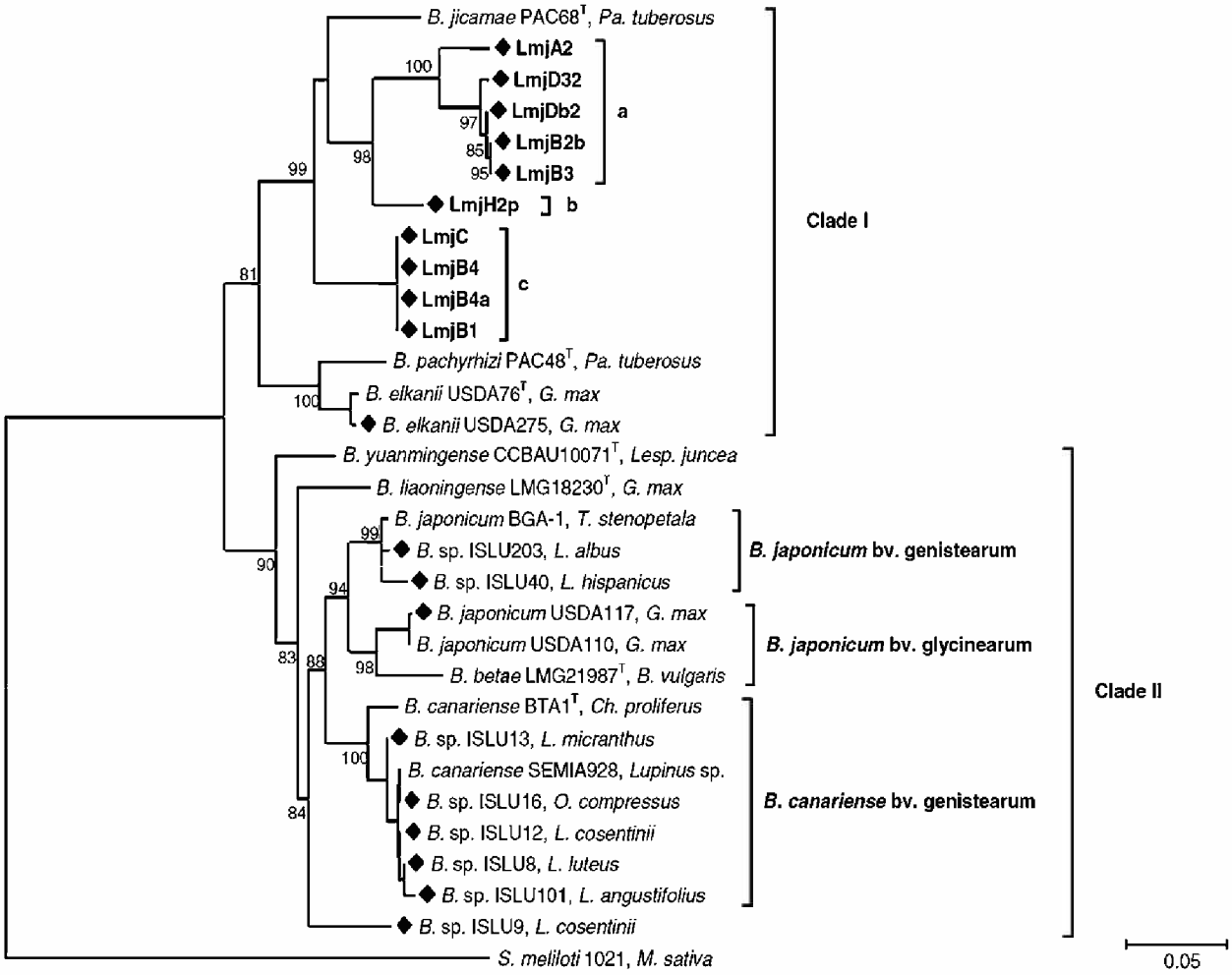

Figure 2. Maximum likelihood phylogenetic tree based on the alignment of a 1 399 bp concatenated nucleotide sequence of *glnII* (522 bp), *recA* (434 bp) and *atpD* (443 bp). ML bootstrap support values (≥ 80% over 1 000 replicates) are indicated at the relevant nodes. Isolates of *L. mariae-josephi* are shown in boldface type. Legume hosts follow the strain names. Gene sequences of bradyrhizobial strains preceded by diamonds were obtained in this study. The scale bar shows the number of substitutions per site. Letters 'a', 'b' and 'c' reflect different groups of *L. mariae-josephi* isolates. Abbreviations are defined in the legend for Fig. 1.

L. mariae-josephi isolates based on *nodC* is consistent with their host specificity range (ie. unable to nodulate *Lupinus* spp. adapted to acid soils such as *L. angustifolius* or *L. luteus*), and again suggests that it is the plant host that determines endosymbiotic bacterial selection.

Additional isolates and detailed phenotypic and genetic studies will be required to clearly define the biodiversity of Lmj bacteria. The allopatric (geographic) speciation of Lmj bradyrhizobia may have resulted from the colonization of the singular habitat (basic and high calcium carbonate soils) of this Spanish area by its unique legume host.

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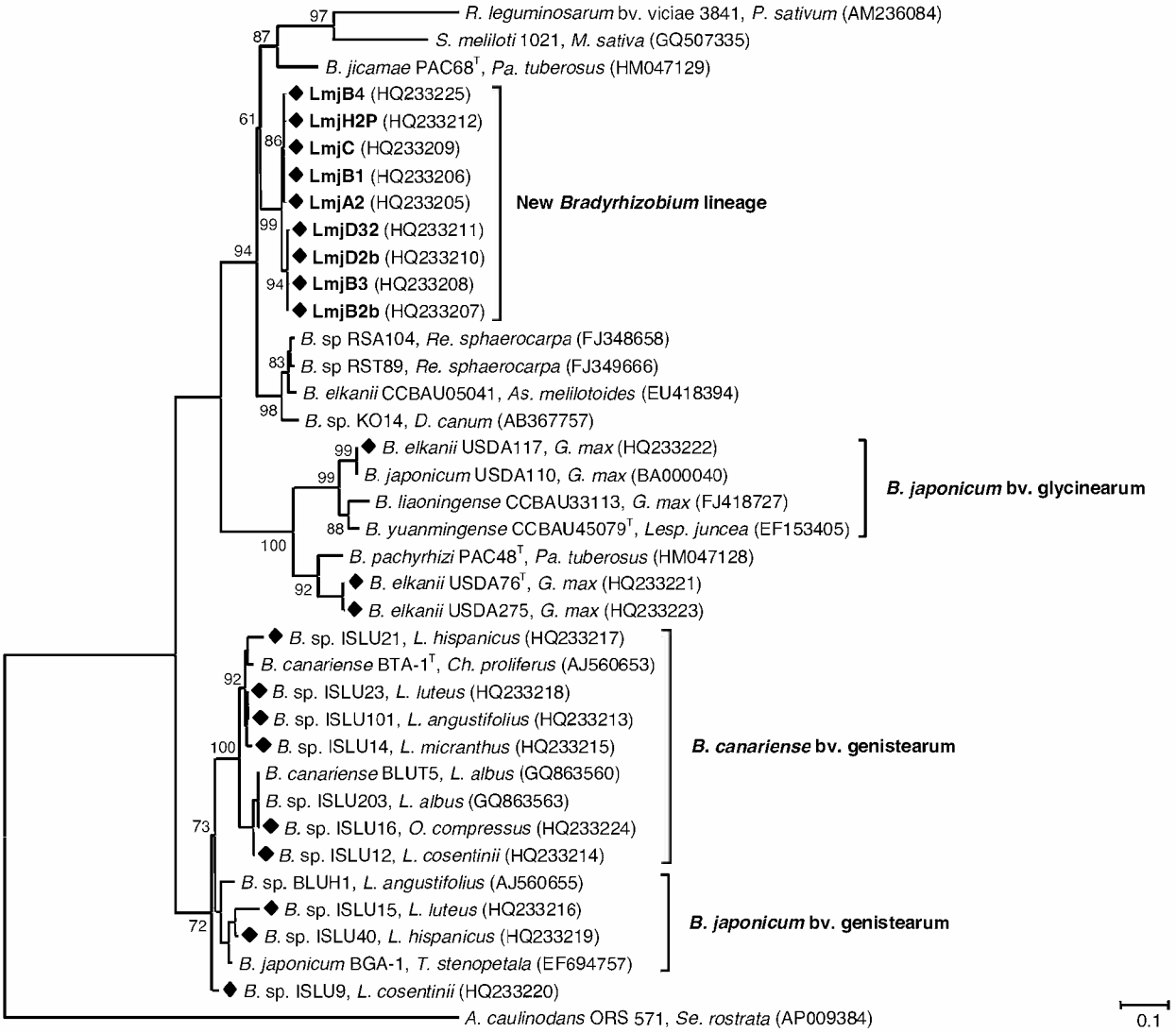

Figure 3. Maximum likelihood phylogenetic tree based on *nodC* sequences (455 bp). Isolates designated Lmj have been isolated from *L. mariae-josephi* nodules in this study and are shown in boldface type. Bootstrap values (≥60% over 1 000 replicates) are indicated at the relevant nodes. Legume hosts follow the strain names. Accession numbers from GenBank are shown in brackets. Isolates of *L. mariae-josephi* and strains indicated with diamonds were sequenced in this study. The scale bar shows the number of substitutions per site. Abbreviations are defined in the legend for Fig. 1.

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